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Antibodies that perturb signal transduction of cancer cells have demonstrated significant utility in the treatment of breast cancer and lymphoma. As signal transduction in the Epidermal Growth Factor Receptor (EGFR) family (EGFR, HER2, HER3, HER4) involves ligand binding and subsequent heterodimerization of two members, the most potent monoclonal antibody (MAb)-based agent would likely be one that also mediates a similar crosslinking event. The fundamental hypothesis underlying this Concept Award Project was that signal transduction through components of the EGFR family could be manipulated through the construction of novel bispecific antibodies that engage multiple epitopes of this family. The goals of this proposal were to develop a novel, rapid methodology to create bispecific single-chain Fv (bs-scFv) molecules using molecular shuffling of two large groups of

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Introduction

Antibodies that perturb signal transduction of cancer cells have demonstrated significant utility in the treatment of breast cancer and lymphoma. As signal transduction in the Epidermal Growth Factor Receptor (EGFR) family (EGFR, HER2, HER3, HER4) involves ligand binding and subsequent heterodimerization of two members, the most potent monoclonal antibody (MAb)-based agent would likely be one that also mediates a similar crosslinking event. ***The fundamental hypothesis underlying this Concept Award Project was that signal transduction through components of the EGFR family could be manipulated through the construction of novel bispecific antibodies that engage multiple epitopes of this family.*** The goals of this proposal were to develop a novel, rapid methodology to create bispecific single-chain Fv (bs-scFv) molecules using molecular shuffling of two large groups of scFv (libraries) specific for HER2 and HER3 and to perform preliminary evaluations of the in vitro specificity and anti-tumor effects against cells that over express both target antigens.

Body

Research Accomplishments:

Task 1. Isolate scFv clones from a large human scFv library that are reactive with HER3. We used human HER3 extracellular domain (ECD) as a target to pan a phage display library. A number of scFv were isolated that bind to HER3 (Table 1). PCR fingerprinting revealed that 6 contained unique sequences. Note: while it was not directly identified in the scope of the proposal, we also isolated scFv molecules that bound to HER4 ECD (10 unique clones) and EGFR ECD (33 unique clones).

Table 1. Properties of scFv sublibraries.

Antigen	# of scFv libraries screened	# Unique scFv clones in sublibrary
HER2	2	2*
HER3	1	6
HER4	1	10
EGFR	3	33

*Note, 75 affinity mutants ranging in affinity from the mM to pM level have been created from one of these clones

Task 2. Create bs-scFv molecules using scFv shuffling. A unique peptide spacer with the amino acid sequence "NSGAGTSGSGASGEGSGSKL" was designed that would impart flexibility and resistance to serum proteases. Based upon incompatible restriction sites between the scFv clones, scFv shuffling techniques will require significant sight-directed mutagenesis to alter restriction sites. We have begun this process. In the meantime, we opted to select a few anti-HER3 scFv molecules and use these together with the peptide spacer (shown above) and our best anti-HER2 scFv (ML3.9) to create a few bs-scFv for the initial screening process. As a result of this effort, we have now created two bs-scFv that are specific for HER2 and HER3 (ALM and FLM), two homodimeric scFv that are specific for HER3 (ALA and FLF), one homodimeric scFv that is specific for HER2 (MLM) and one heterodimeric scFv that is specific for two epitopes on HER3 (FLM).

Task 3. Evaluate the specificity of the bs-scFv molecules for both target antigens. The specificity of the first bs-scFv, ALM, for HER2 and HER3 ECDs was evaluated by surface plasmon resonance on the BIAcore instrument. It was found to be capable of binding to HER2 ECD, HER3 ECD and of forming a complex between HER2 and HER3 ECDs in a sandwich assay (Figures 1-3).

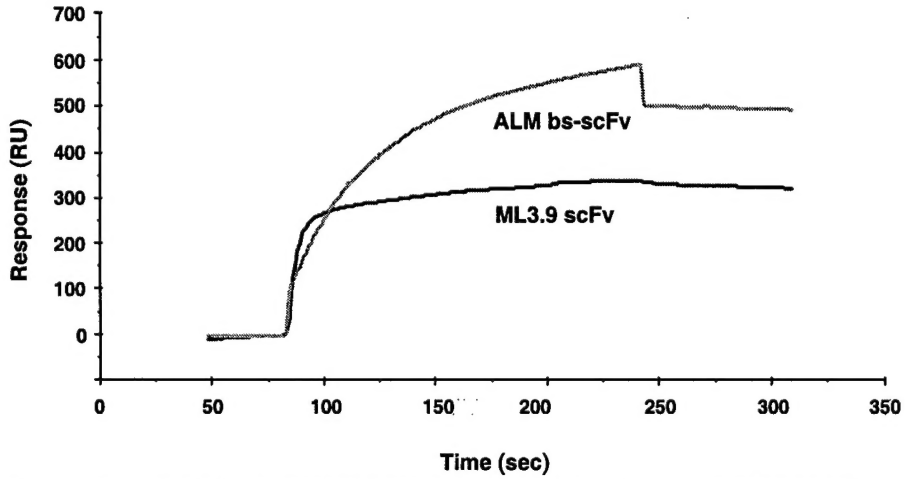

Figure 1. ALM (anti-HER2/HER3 bs-scFv) binding to HER2 ECD compared with the binding of the parent anti-HER2 scFv, ML3.9.

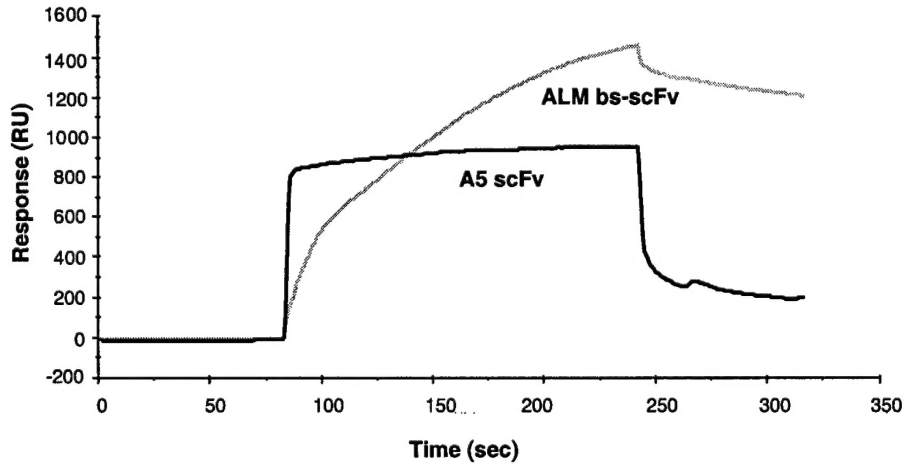

Figure 2. ALM (anti-HER2/HER3 bs-scFv) binding to HER3 ECD compared with the binding of the parent anti-HER3 scFv, A5.

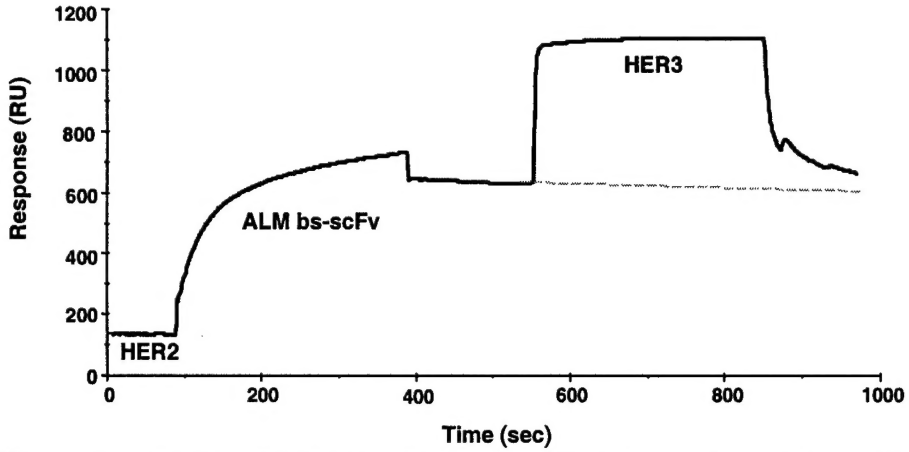

Figure 3. ALM (anti-HER2/HER3 bs-scFv) binding to immobilized HER2 ECD followed by HER3 ECD in solution in a sandwich assay.

Task 4. Evaluate the function of the bs-scFvs. The functional consequences of the binding of first bs-scFv, ALM, to cells that overexpress HER2 and HER3 was evaluated in MTT assays, a clonogenicity assay and by flow cytometry. In a five day MTT assay, which is a measure of impact on cellular proliferation, ALM mediated a similar degree of growth inhibition of human breast tumor BT-474 cells as did Herceptin – a clinically approved MAb that targets HER2 (Figure 4). In a 17 day clonogenicity assay, ALM incubated with BT-474 breast cancer cells at a roughly equimolar concentration to the amount of cell-surface HER2 lead to approximately a 50% reduction in tumor cell survival (Figure 5). Increasing the quantity lead to a decrease in efficacy. Finally, the impact of ALM on HER2 and HER3 expression on BT-474 cells was determined by flow cytometry. It was found that 1.6 ug/ml ALM incubated with 1.6 x 10^6 BT-474 cells resulted in a reduction of cell-surface HER2 after 4 hr @ 37°C and a reduction in cell surface HER3 after 48 hr @ 37°C (Figure 6).

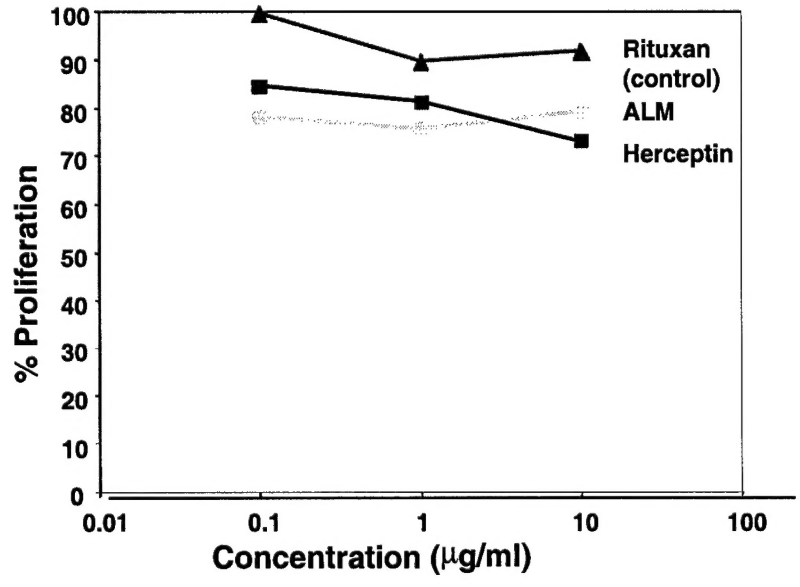

Figure 4. In an MTT assay, ALM has a similar anti-proliferative effect on human breast tumor BT-474 cells as does Herceptin. Rituxan is included as a negative control.

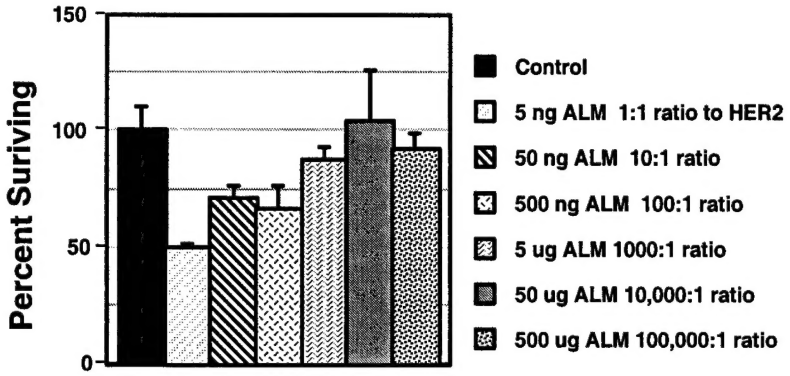

Figure 5. Results of a 17 day clonogenicity assay using increasing concentrations of ALM with BT-474 cells. Ratios of ALM to HER2/*neu* are indicated, n=3 plates/concentration.

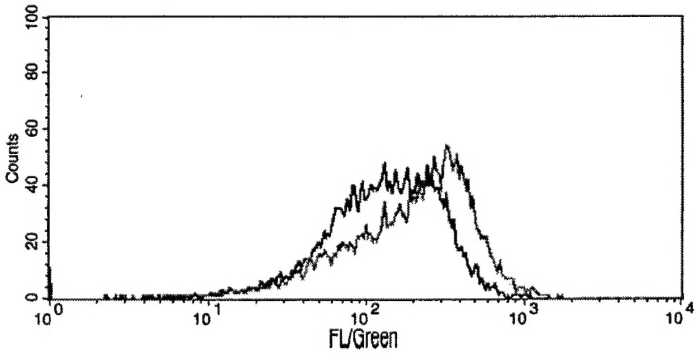

Figure 6. Impact of ALM on the expression of HER2 at 4 hrs (top) and HER3 at 48 hrs (bottom) on the surface of BT-474 cells. The left-hand peaks on each plot are with ALM, the right-hand peaks are without ALM. 1.6 ug/ml ALM incubated with 1.6 x 10^6 BT-474 cells, HER2 detected with 520C9 MAb and HER3 detected with AB4 (Neomarkers).

Key Research Accomplishments

- Isolated scFv from a human phage display library that are specific for HER3, HER4 and EGFR. 6 unique clones that bind to HER3, 10 unique clones that bind to HER4 and 33 unique clones that bind to EGFR.
- Developed a novel peptide spacer that is expected to be protease resistant for use in linking the scFv into a bispecific format (Spacer sequence = NSGAGTSGS GASGEGSGSKL)
- Created the following scFv-based molecules; two bs-scFv that are specific for HER2 and HER3 (ALM and FLM), two homodimeric scFv that are specific for HER3 (ALA and FLF), one homodimeric scFv that is specific for HER2 (MLM) and one heterodimeric scFv that is specific for two epitopes on HER3 (FLM).
- Evaluated the impact of the lead bs-scFv molecule, ALM, on the in vitro growth properties of the human breast tumor cell line, BT-474, that overexpresses HER2 and HER3.
  - In an MTT assay evaluating the impact on cell proliferation, a five day incubation with ALM, Herceptin (positive control) or Rituxan (negative control) lead to comparable inhibition of proliferation with ALM and Herceptin (Figure 4).
  - In a clonogenicity assay, a 17 day incubation with 5 ng to 500 ug concentrations of ALM (ALM to cell surface HER2 ratios of 1:1 to 100,000:1) lead to approximately 50% reduction in cell viability in the group treated with the lowest concentration of ALM (Figure 5). Interestingly, as the concentration was increased, the impact on survival decreased.
  - Have determined by flow cytometry that incubation of BT-474 cells with ALM leads to decreases in the cell surface expression of HER2 and HER3 (**Figure 6**).

Reportable Outcomes

- American Association for Cancer Research abstract. #4810. Production and Evaluation of Bispecific single-chain Fv molecules that target HER2/neu and HER3. E. Horak, L. Shahied, C. Shaller, A. Tesfaye, H. Simmons, R. Alpaugh, N. Greer, T. Heitner, J. Garrison, J. Marks, L. Weiner and G. Adams.Proc. AACR. 43:971, 2002.
- Presentation at "AntibOZ", An International Forum: Predicting the Next Wave of Protein-Based Therapies and Immunodiagnostics. Heron Island, Queensland Australia. April 8-12th, 2002.
- Provisional Patent filed April 12, 2002.

Conclusions

The generation of a scFv shuffling system for creating multiple bs-scFv partners was more difficult than expected. While progress has been made in this area, we have proceeded to make a two of bs-scFv molecules that target HER2 and HER3 and homodimeric controls that target HER2 or HER3. The lead molecule, ALM, has been evaluated for specificity and efficacy in vitro. We have found that it binds specifically to both targets and mediates a decrease in cell surface expression of HER2 and HER3 and leads to a reduction in cell survival and proliferation. We plan to evaluate the in vivo efficacy of ALM in immunodeficient mice bearing s.c. human breast cancer tumors. If

ALM shows a similar effect in vivo, we will plan on initiating clinical trials as soon as possible.

References

N/A

Appendices

American Association for Cancer Research abstract. #4810. Production and Evaluation of Bispecific single-chain Fv molecules that target HER2/neu and HER3. E. Horak, L. Shahied, C. Shaller, A. Tesfaye, H. Simmons, R. Alpaugh, N. Greer, T. Heitner, J. Garrison, J. Marks, L. Weiner and G. Adams.Proc. AACR. 43:971, 2002.

#4808 Glycopeptide-Related Surface Antigen (GRSA) appears to be a marker of breast ductal carcinoma in situ. William G. North, Brendan P. Keegan, Vincent A. Memoli, and Wendy A. Wells. *Dartmouth Medical School, Lebanon, NH.*

We previously have shown that expression of the vasopressin gene by breast cancer gives rise, not only to biologically active peptide also, to protein products at the cell surface referred to by us as glyc lated surface antigen (GRSA). This name was coined because the ts with antibodies raised against the C-terminal half (18 AA) opeptide moiety of provasopressin. Rabbit polyclonal antibodies sly employed to show by immunohistochemistry (and by Western t GRSA was present in all of 23 breast cancers examined, but w m 21 cases of breast fibrocystic disease (including atypical d plasia) and from normal breast tissue. We now report that GRSA n almost all (>90%) of 38 cases of cytologically identified ductal n situ (DCIS) examined using both our protein A-purified polyclona and a new monoclonal antibody (MAG 1). MAG 1 is a mouse IgG1 enerated by us using hybridoma technology and purified through nity chromatography from ascites fluid. Immunohistochemistry ed on 3-5 mm sections of AMEX-fixed or formaldyhyde-fixed tiss ed cytologically for the presence of DCIS. Human anterior hyp was employed as a positive control and antibody blocked with per as a negative control. Antigen-retrieval was performed on form d tissues by treating sections with trypsin for 10 min at ambient tempe over 90% DCIS examined were positive for GRSA and this staining was det ined to be specific since it was entirely blocked in the presence of peptide antigen. Our data indicate GRSA is a marker of early oncogenic transformations in breast tissues and that MAG 1 can be used as an effective marker of oncogenic transformation in the breast. Identification of GRSA may be useful in discriminating DCIS from ADH, and identifying DCIS cytologically.

#4809 Detection of HPV-infected cells utilizing an on-chip hydrodynamic focusing system. Lauren S. Gollahon, Zhiqiang Du, Nikita Patel, Marc Pimsleur, Diana Contreras, and Darryl Bornhop. *Texas Tech University, Lubbock, TX, and Texas Tech University - Health Sciences Center, Lubbock, TX.*

Cervical cancer is the most common malignant reproduct cancer in women from 15 to 35 years of age. It is the third cause of deat cer in women behind lung and breast cancer. It is estimated that o ber of invasive cervical cancer cases diagnosed in the U.S. for 2002 will eventually die of the condition. Annually, 35,000 women are diagr early stage cervical cancer. The progression of disease is directly c o infection by certain strains of human papillomavirus (HPV). Early de critical for the successful therapy of cervical cancer, therefore it is im identify abnormal cells as quickly and accurately as possible. The m em is accurate identification of early stage cervical intraepithelial nec N) from collected cells. Cervical samples are routinely collected by Pa and microscopically examined for pathologic features. To date, ther accurate or convenient method of screening for HPV infected cells st fixing the cells and staining the cell preparation. Identification of H on is performed through ancillary techniques that rely primarily or and screening for cytoplasmic or nuclear proteins or through DNA a herefore, there is a need for novel methods of cell isolation, particular d to the capture of abnormal cells. We have applied an antibody- drodynamic focusing microflow system for the capture and enrichr V-infected human cervical epithelial cells. This new antibody-based c e and enrichment system allows sensitive and accurate detection sence of HPV in targeted cells without secondary manipulations t ples. Results show human cervical epithelial (HCE) cells containing ere captured from mixed populations of infected and uninfected HCE HCE and human cervical stromal (HCS) cells. We are currently modifying this chnique for applications to other target specific exfoliated cells from large volumes and/or bodily fluid flushes, e.g. pancreatic brushes, colonic effluents and ductal lavage.

#4810 Production and evaluation of bispecific single-chain Fv molecules that target HER2/neu and HER3. Eva M. Horak, Lillian S. Shahied, Calvin C. Shaller, Abohawariat Tesfaye, Heidi H. Simmons, R. Katherine Alpaugh, Nathaniel B. Greer, Tara Heitner, Jennifer L. Garrison, James D. Marks, Louis M. Weiner, and Gregory P. Adams. *Fox Chase Cancer Center, Philadelphia, PA, and University of California, San Francisco, San Francisco, CA.*

Signal transduction through members of the EGFR Family (EGFR, HER2/neu, HER3 and HER4) is dependent upon the formation of homodimers or heterodimers triggered by the binding of ligand. Overexpression of the members of this receptor family has been correlated with a poor prognosis in a number of types of cancer. Antibodies, such as Herceptin (anti-HER2), C225 and ABX-EGF (anti-EGFR) or small molecules like IRESSA, that perturb signaling through these receptors, have been associated with significant clinical responses.We hypothesize that bispecific single-chain Fv (bs-scFv) that bind to selected pairs of these receptors could prevent ligand induced signaling and trigger cytostatic or cytotoxic effects. We have previously described the production of the extracellular domains of EGF, HER2/neu, HER3 and HER4 receptors and their use as targets for selection of specific binders from a naive human scFv phage display library. We are now producing single gene bs-scFv molecules from these four sublibraries with the goal of functionally selecting bispecific scFv molecules that target epitope pairs and mediate anti-tumor effects. Our initial focus has been on HER2/neu and HER3. The first bs-scFv we have produced, A5-ML3.9, is capable of binding to both the HER3 and HER2/neu receptors. Its *in vitro* binding kinetics, cell surface retention, *in vivo* tumor-targeting properties and ability to impact the growth of tumor cells expressing both HER2/neu and HER3 receptors will be presented. This research was supported by Concept Award # DAMD17-01-1-0520 from the Department of Defense Breast Cancer Research Program.

#4811 Synthetic immunostimulatory oligonucleotides show anti-tumor activity and increase therapeutic effectiveness of monoclonal antibody and chemotherapeutic agents. Hui Wang, Jie Hang, Mao Li, Zhenqi Shi, Lin Lin, Ekambar R. Kandimalla, Dong Yu, Qiuyan Zhao, Sudhir Agrawal, and Ruiwen Zhang. *Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL, and Hybridon, Inc., Cambrid MA.*

It has been suggested that the observed antitumo es of antisense oligonucleotidess that contain CpG dinucleotides co a combination of antisense and immune-mediated effects. Oli s containing CpG dinucleotides in certain sequence contexts induce a number of cytokines, including IL-12, IL-6, IFN-γ, and presence of CpG dinucleotides in antisense oligonucleotides c to the antitumor activity of antisense agents independent of d n of targeted gene product. In the present study, we used sever os containing natural and synthetic immunostimulatory motifs to e antitumor activity in human cancer xenograft models. The s administration of these synthetic CpG oligos at various dose umor growth in mice bearing xenografts of human cancers of c and DLD-1) and prostate (DU145) and glioblastoma (U87MG). T ic CpG oligos also increased therapeutic effects of monoclonal ar xan and chemotherapeutic agent, paclitaxel in all three models tested (, DLD-1 and DU145). These data demonstrate that the oligonucleotides tr.at contain CpG dinucleotides exhibit anticancer activity mainly by immune stimulation. In addition, we demonstrated that synthetic motifs incorporated in oligonucleotides influence the immunostimulatory activity of CpG oligos, resulting in an improvement of host side effects. This study provides a basis for future development of synthetic immunostimulatory oligonucleotides as cancer therapeutic agents used alone or in combination with conventional therapies. (Supported by NIH/NCI grant CA 80698.)

#4812 Combined reovirus and 1,3-bis (2-chloroethyl)-1-nitrosourea therapy against murine hematopoietic tumors. Timothy A. Steele. *Des Moines University-Osteopathic Medical Center, Des Moines, IA.*

Reovirus therapy of tumors is an emerging area of study as evidenced by recent investigations (Coffey, et al., Science 282:1332-1334, 1998 and Steele and Cox, Cancer Biother. 10:307-315, 1995). In our laboratory, EL-4 or L1210 tumor-bearing B6D2F1 mice treated at day 4 (following intraperitoneal tumor injection) with 9 mg/kg 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU) ed at day 6 with 1 x 109 plaque-forming units of reovirus type 3 (Dearing ted in 65-80% of the mice being cured of tumor. Tumor-bearing with reovirus or BCNU alone typically yielded 0% and 20% sur tively. Challenge of cured mice with homologous tumor yielded val, whereas challenge with heterologous tumor produced 0% sur ting an immune-mediated tumor elimination mechanism. That the ay proceed via an immune-mediated mechanism was strengthe finding that daily *in vivo* administration of cyclosporine, an immu ve drug, to therapy-treated animals abrogated the survival effect. etric analysis revealed an increase in natural killer cells, macroph 8+ lymphocytes in the mesenteric nodes of therapy-treated mice untreated or tumor-bearing control mice. In addition, an increase er of spleen macrophages was also observed in therapy-treated m ed to untreated or tumor-bearing control mice. Since reovirus inducer of α/β interferons in mice, we investigated whether mur inant interferon-α (IFN-α) could replace reovirus in the therapy. I IFN-α, varying between 500 - 10,000 units, were able to produ gnificantly ($p<0.05$) above control mice. However, only the 10,0 e could produce cures equivalent to reovirus. Challenge of cured ir r reovirus-treated mice with EL-4 tumor yielded 100% survival, wh challenge with heterologous tumor (L1210) produced 0% survival, suggesti g that tumor elimination proceeded via an immune-mediated mechanism. Intraperitoneal injection of anti-IFN-α antibodies did not diminish the effect of the therapy, suggesting the presence of redundant cytokines. Further studies are warranted to investigate the potential of combined reovirus and chemotherapy on hematopoietic tumors.

#4813 IL-6 and interferon-γ levels following chem breast cancer. Andrea M. Mastro, Nancy I. Williams, Jennif uener, Elizabeth Orsega-Smith, William J. Kraemer, Aaron chard H. Dixon, Judy Underwood, Mary Miles, and Kate Wag niversity, University Park, PA, University of Connecticut, S nger, State College, PA, CMSA, State College, PA, and Mon ersity, Bozman, MT.

In a study designed to follow of CD4+ T lymphocytes following chemotherapy for breast c ed if there were changes in interferon gamma (IFNg), a Th1 nterleukin 6 (IL6), a Th2 cytokine. These cytokines were mea SA in the plasma of women at the following times: prechemothera stchemotherapy, postradiation and 3 and 6 months